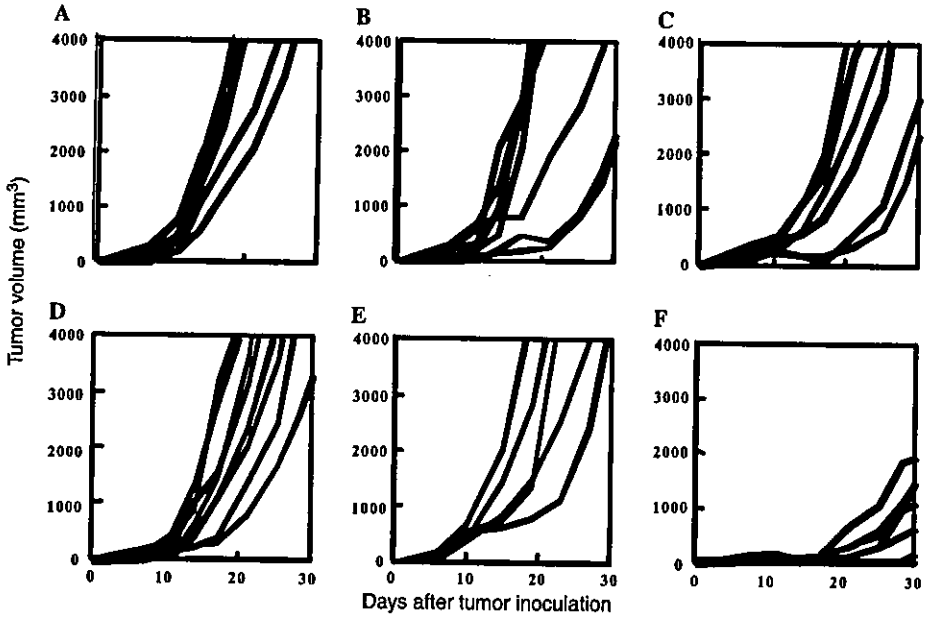

Fig. 6. In vivo immunization with pCMV-script/OVA-FL elicits protective immunity against OVA-expressing tumor. C57/BL6 mice were immunized twice at two-week intervals with saline control (A), 50 μg of naked pCMV-script/OVA (B), Lipofectin/pCMV-script/OVA (5 μg) complex (C), pCMV-script/OVA (5 μg) encapsulated by a conventional liposome (D), pCMV-script/LacZ (5 μg) containing FL (E), or pCMV-script/OVA (5 μg) containing FL (F). All mice were challenged intradermally in the abdomen with 1×10^6 live OVA-expressing EG7 cells four weeks after the last immunization and survival was monitored.

Acknowledgments

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Optimal site-specific PEGylation of mutant TNF-α improves its antitumor potency

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Abstract

Recently, we created a lysine-deficient mutant tumor necrosis factor-α [mTNF-α-Lys(−)] with full bioactivity in vitro compared with wild-type TNF-α (wTNF-α), and site-specific PEGylation of mTNF-α-Lys(−) was found to selectively enhance its in vivo antitumor activity. In this study, we attempted to optimize this PEGylation of mTNF-α-Lys(−) to further improve its therapeutic potency. mTNF-α-Lys(−) was site-specifically modified at its N-terminus with linear polyethylene glycol (LPEG) or branched PEG (BPEG). While randomly mono-PEGylated wTNF-α (ran-$LPEG_{5K}$-wTNF-α) with 5 kDa of LPEG ($LPEG_{5K}$) had about only 4% in vitro bioactivity of wTNF-α, mono-PEGylated mTNF-α-Lys(−) [sp-PEG-mTNF-α-Lys(−)] with $LPEG_{5K}$, $LPEG_{20K}$, $BPEG_{10K}$, and $BPEG_{40K}$ had 82%, 58%, 93%, and 65% bioactivities of mTNF-α-Lys(−), respectively. sp-LPEG-mTNF-α-Lys(−) and sp-$BPEG_{10K}$-mTNF-α-Lys(−) had much superior antitumor activity to those of both unmodified TNF-αs and ran-$LPEG_{5K}$-wTNF-α, though sp-$BPEG_{40K}$-mTNF-α-Lys(−) did not show in vivo antitumor activity. Thus, the molecular shape and weight of PEG may strongly influence the in vivo antitumor activity of sp-PEG-mTNF-α-Lys(−).

Tumor necrosis factor-α (TNF-α), an antitumor cytokine, has numerous bioactivities such as direct cytotoxicity against tumor cells, activation of immune antitumor response, and selective impairment of tumor-blood vessels [1]. Thus, TNF-α has been considered as a novel antitumor agent [2,3]. However, very high doses of TNF-α as a systemic antitumor agent were required to obtain the sufficient clinical responses, since TNF-α is rapidly cleared from the circulation and is widely distributed to various tissues after its intravenous (i.v.) administration. As a result, TNF-α with pleiotropic in vivo actions exhibited unexpected toxic side-effects, typified by pyrexia and hypertension [4,5]. Its systemic application has been abandoned despite intratumoral administration of TNF-α showing significant antitumor effects in phase I studies [6]. Presently, the clinical application of TNF-α is limited to intratumoral administration, despite its high expectations as a systemic agent. These in vivo drawbacks of TNF-α are also found in the clinical applications of other bioactive proteins [7]. Thus, the development of a drug delivery system (DDS) is necessary for the promotion of protein therapies following recent marked advances in proteomics and structural genomics.

PEGylation has been known as one of the most useful DDS for optimizing protein therapies [8,9]. The PEGylation of proteins increases their molecular size and steric hindrance, both of which are derived from polyethylene glycol (PEG) attached to bioactive proteins, resulting in augmented plasma half-lives and

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in vivo stability. Due to these comprehensive effects of PEGylation, PEGylation of proteins could enhance therapeutic potency and could reduce undesirable effects. We also reported that the PEGylation of proteins such as TNF-α, superoxide dismutase, interleukin-6, and immunotoxin could enhance therapeutic potency and reduce undesirable side effects [10–16]. However, the PEGylation of proteins is mostly non-specific and targeted at all the lysine residues in the protein, some of which may be in or near an active site. As a result, PEGylation of proteins was accompanied by a significant loss of their specific activities in vitro. For instance, randomly mono-PEGylated interferon α2a ($BPEG_{40K}$-IFN-α2a), which is IFN-α2a, conjugated with 40 kDa of branched PEG, has been clinically used for the treatment of hepatitis C. The $BPEG_{40K}$-IFN-α2a, which is a mixture of various positional isomers, had about 10% of its bioactivity compared to unmodified IFN-α2a [17,18]. Thus, clinical application of PEGylated proteins has been limited in most cases except for some bioactive proteins, such as IFN-α.

To overcome this problem of PEGylation, we have developed a novel strategy for site-specific mono-PEGylation using TNF-α for the improvement of its in vivo antitumor potency [16]. We isolated a lysine-deficient mutant TNF-α with full bioactivity [mTNF-α-Lys(−)] from phage libraries expressing mTNF-αs, in which all lysine residues were replaced with other amino acids. In the mTNF-α-Lys(−) molecule, Lys11, Lys65, Lys90, Lys98, Lys112, and Lys128 of wTNF-α were replaced with Met11, Ser65, Pro90, Arg98, Asn112, and Pro128, respectively. This mTNF-α-Lys(−) was site-specifically mono-PEGylated at its N-terminus with 5 kDa of linear PEG ($LPEG_{5K}$). This site-specifically mono-PEGylated mTNF-α showed increased antitumor therapeutic potency, compared to an unmodified wild-type TNF-α (wTNF-α) and a randomly mono-PEGylated wTNF-α. In this study, to optimize the site-specific PEGylation at the N-terminus of mTNF-α-Lys(−) in order to further improve its therapeutic potency, mTNF-α-Lys(−) was modified with LPEG and BPEG, which had different molecular weights (Mw). Additionally, through molecular modeling of the complexes between wTNF-α and TNF receptor-I (TNF-RI), we discussed why mTNF-α-Lys(−) had comparable in vitro bioactivity to wTNF-α, despite reports that some lysine residues were essential for its bioactivity. This study will provide the information necessary to optimally design a PEGylated TNF-α suitable for therapeutic use as a systemic antitumor agent.

Materials and methods

Expression and purification of TNF-αs. Plasmids pYas1-TNF and pYas-mTNF encoding human wTNF-α and mTNF-α-Lys(−), respectively, under the control of a T7 promoter, were prepared. wTNF-α and mTNF-α-Lys(−) proteins were produced in *Escherichia coli* BL21(DE3) harboring the expression plasmid pYas1-TNF and pYas-mTNF as described [14,16]. mTNF-α-Lys(−) had K11M, K65S, K90P, K98R, K112N, and K128P compared with wTNF-α. Endotoxin levels were determined to be <300 pg/mg each in the wTNF-α and mTNF-α-Lys(−).

PEGylation of mTNF-α-Lys(−). As shown in Fig. 1, activated LPEGs with Mw of 5 and 20 kDa ($LPEG_{5K}$, $LPEG_{20K}$) and activated BPEGs with Mw of 10 and 40 kDa ($BPEG_{10K}$, $BPEG_{40K}$) were purchased from Shearwater Polymers (Huntsville, AL). mTNF-α-Lys(−) in PBS was reacted with 50 times molar excess of $LPEG_{5K}$ and $LPEG_{20K}$ as well as 250 times molar excess of $BPEG_{10K}$ and $BPEG_{40K}$ against total primary amine groups of mTNF-α-Lys(−) at 37 °C for 30 min. wTNF-α in PBS was reacted with five times molar excess of $LPEG_{5K}$ against total primary amine groups of wTNF-α at 37 °C for 30 min. Site-specifically mono-PEGylated mTNF-α-Lys(−)s and randomly mono-PEGylated wTNF-α were purified by anion-exchange and gel filtration chromatographies. The specific bioactivities of mono-PEGylated forms were examined by a cytotoxicity assay using LM cells, a cell line derived from L929 cells. Kinetic and equilibrium constants for the interaction between mono-PEGylated TNF-αs and TNF-RI were measured using surface plasmon resonance in a BIAcore 2000, as described.

Antitumor studies. All experimental protocols for animal studies were in accordance with the *Guide for Laboratory Animal Facilities and Care* (NIH publication 85-23, revised 1985). These protocols have been approved by the Committee of the Pharmaceutical School, Osaka University. The antitumor effects of mTNF-α-Lys(−) and sp-PEG-mTNF-α-Lys(−)s were evaluated in mice bearing Meth-A fibrosarcoma. Meth-A cells were implanted intradermally (2×10^5 cells/site) in 5-week-old female BALB/c mice. On day 7, when the tumor diameter reached 7 mm, TNF-α molecules were administered by a single i.v. injection. Antitumor potency was estimated from the tumor volume and tumor hemorrhagic necrosis within 24 h of the injection.

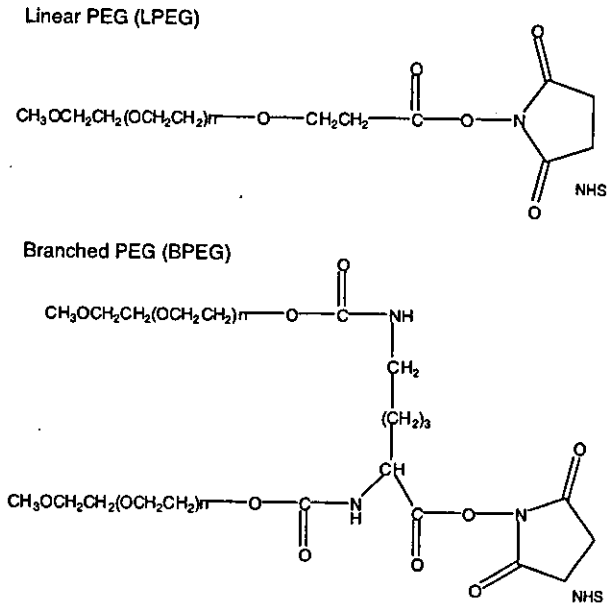

Fig. 1. Chemical structure of activated PEG molecules.

Results

Site-specific PEGylation of mTNF-α-Lys(−)

As shown in Fig. 1A, $BPEG_{10K}$ and $BPEG_{40K}$ consist of two $LPEG_{5K}$ molecules or two $LPEG_{20K}$ molecules, respectively. wTNF-α and mTNF-α-Lys(−) were modified with these activated PEGs. PEGylated TNF-α molecules were detected by SDS–PAGE using a Coomassie blue staining (data not shown). As a result, we found that a single PEG molecule was site-specifically attached to the N-terminus of mTNF-α-Lys(−), whereas a number of $LPEG_{5K}$ molecules were attached to wTNF-α at random. Site-specifically mono-PEGylated mTNF-α-Lys(−)s appeared to have a higher molecular size (Ms) than calculated Ms when compared to the Mw marker proteins, probably due to the high mobility of attached PEG in aqueous solution and its formation of a highly hydrated shield around mTNF-α-Lys(−). To examine the usefulness of site-specifically mono-PEGylated mTNF-α-Lys(−)s, randomly mono-PEGylated wTNF-α, which was composed of positional isomers with PEG at various sites, was separated and purified by anion-exchange and gel filtration chromatographies.

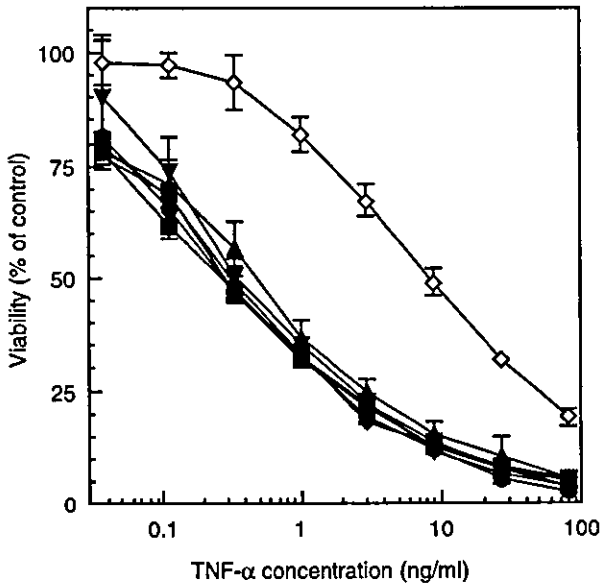

Fig. 2. In vitro bioactivity of mono-PEGylated forms of TNF-αs. The specific activity of mono-PEGylated forms of TNF-α was measured by cytotoxic assay using LM cells in the presence of actinomycin D. Each data value represents means ± SD. □, wTNF-α; ◇, ran-$LPEG_{5K}$-wTNF-α; ■, mTNF-Lys(−); ◆, sp-$LPEG_{5K}$-mTNF-α-Lys(−); ●, sp-$LPEG_{10K}$-mTNF-α-Lys(−); ▲, sp-$LPEG_{20K}$-mTNF-α-Lys(−); and ▼, sp-$LPEG_{40K}$-mTNF-α-Lys(−).

In vitro bioactivity of sp-PEG-mTNF-α-Lys(−)s

To assess the relationship between the shape of PEG with various Mw and in vitro bioactivity of sp-PEG-mTNF-α-Lys(−)s, we assessed the specific bioactivity of sp-PEG-mTNF-α-Lys(−)s using LM cells in the presence of actinomycin D at a concentration of 2 μg/ml (Fig. 2 and Table 1). While the remaining bioactivity (LC50) of ran-$LPEG_{5K}$-wTNF-α was only 4% compared with that of wTNF-α, all sp-PEG-mTNF-α-Lys(−)s had much higher bioactivity than ran-$LPEG_{5K}$-wTNF-α. Additionally, the remaining bioactivities (LC50) of sp-$BPEG_{10K}$-mTNF-α-Lys(−) [93% compared with that of mTNF-α-Lys(−)] or sp-$BPEG_{40K}$-mTNF-α-Lys(−) [65% compared with that of mTNF-α-Lys(−)] were similar to those of $LPEG_{5K}$-mTNF-α-Lys(−) [82% compared with that of mTNF-α-Lys(−)] or sp-$LPEG_{20K}$-mTNF-α-Lys(−) [58% compared with that of mTNF-α-Lys(−)], respectively. These results indicated that site-specific mono-PEGylation of mTNF-α-Lys(−) with BPEG, which had two LPEG molecules, may enable the designing of a sp-PEG-mTNF-α-Lys(−) with higher Ms and stronger in vitro bioactivity.

The affinity of sp-PEG-mTNF-α-Lys(−)s for human TNF-RI was measured using BIAcore (Table 1). The affinity of ran-$LPEG_{5K}$-wTNF-α was markedly reduced (20% of wTNF-α), but all sp-PEG-mTNF-α-Lys(−)s retained sufficient affinity for TNF-RI compared with both wTNF-α and mTNF-α-Lys(−). In brief, the off rates (dissociation rates) of sp-PEG-mTNF-α-Lys(−)s

Table 1
Biological activities and binding parameters of various forms of TNF-α

	LC50[a] (ng/ml)	k_{on}[b] ($\times 10^5\ M^{-1}\ s^{-1}$)	k_{off}[c] ($\times 10^{-4}\ s^{-1}$)	k_d[d] ($\times 10^{-10}$ M)
wTNF-α	0.34	19.0 ± 5.4	2.9 ± 0.5	1.7 ± 0.8
ran-$LPEG_{5K}$-wTNF-α	8.69	2.4 ± 0.4	2.0 ± 0.4	8.5 ± 1.1
mTNF-α-Lys(−)	0.28	17.6 ± 5.7	3.7 ± 0.6	2.3 ± 1.1
sp-$LPEG_{5K}$-mTNF-α-Lys(−)	0.34	15.3 ± 5.7	4.6 ± 0.4	3.4 ± 1.6
sp-$BPEG_{10K}$-mTNF-α-Lys(−)	0.30	12.3 ± 5.2	4.4 ± 0.3	3.9 ± 1.3
sp-$LPEG_{20K}$-mTNF-α-Lys(−)	0.48	10.2 ± 4.4	5.0 ± 0.5	5.5 ± 2.5
sp-$BPEG_{40K}$-mTNF-α-Lys(−)	0.43	9.2 ± 4.6	4.6 ± 1.2	5.6 ± 1.8

Each data value represents means ± SD.
[a] LC50 is the concentration of various PEGylated TNF-αs capable of killing 50% of LM cells.
[b,c] The rate constants k_{on} and k_{off} are from BIAcore optical biosensor assays.
[d] The equilibrium dissociation constant k_d is calculated from the ratio of k_{off}/k_{on}.

Table 2
Antitumor effects of wTNF-α, mTNF-α-Lys(−), and sp-LPEG$_{5K}$-mTNF-α-Lys(−)

	Dose (U/mouse)	Complete regression (%)[a]	IR (%)[b]	Hemorrhagic necrosis (mm^2)[c]
PBS	—	0	—	0
wTNF-α	45	0	88	0
mTNF-α	45	0	79	3.5 ± 2.0
Sp-LPEG$_{5K}$-mTNF-α-Lys(−)	30	70	18	14.4 ± 3.9

Mice were used in groups of five.
[a] Complete regression was defined when tumor was not regrown for more than 30 days.
[b] Inhibition rate (%); tumor volume measured on day 20 after tumor implantation and compared with the mean tumor volume in the saline treated group.
[c] Tumor hemorrhagic necrosis was scored 24 h after the injection. Each data value is the mean ± SD.

were about the same as that of mTNF-α-Lys(−), and the decrease of their k_d (equilibrium dissociation constant) resulted from the decrease of their rates (association rates). There was a good correlation between in vitro bioactivities of PEGylated TNF-αs and their k_d values.

Antitumor effects of sp-PEG-mTNF-α-Lys(−)s

The in vivo antitumor effects of various TNF-αs on Meth-A solid tumors were assessed by a single i.v. injection (Table 2). The tumor hemorrhagic necrotic area on intradermally implanted Meth-A fibrosarcoma was assessed 24 h after injection. In mice treated with both unmodified wTNF-α and mTNF-α-Lys(−), hemorrhagic necrosis was not observed at the dose of 45 U/mouse. In contrast, sp-LPEG$_{5K}$-mTNF-α-Lys(−) at a dose of 30 U/mouse showed significant tumor hemorrhagic necrosis, and complete regression was observed in 70% of mice treated. As shown in Fig. 3, sp-LPEG$_{20K}$-mTNF-α-Lys(−)s and sp-BPEG$_{10K}$-mTNF–Lys(−) effectively induced tumor hemorrhagic necrosis compared with

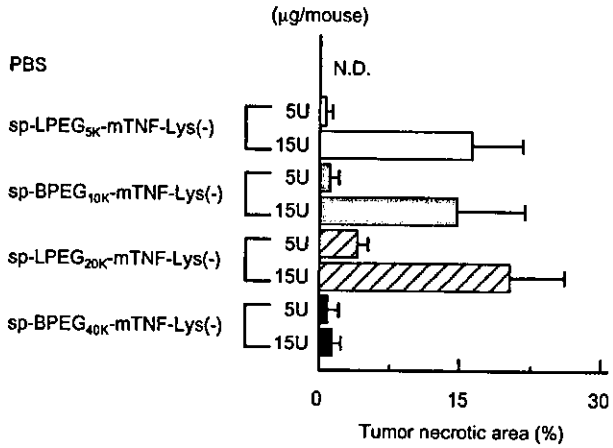

Fig. 3. Antitumor effects of various forms of TNF-α on mice bearing intradermally implanted Meth-A solid tumors. Tumor necrotic effects of i.v.-administered TNF-α and mono-PEGylated TNF-α forms. Tumor hemorrhagic necrosis was scored 24 h after the injection. Mice were used in groups of five. Each data value is the means ± SEM. N.D., not detected.

mTNF-α-Lys(−), but the antitumor effects of sp-BPEG$_{40K}$-mTNF-α-Lys(−) could not be observed.

Discussion

Continuous-infusion or frequent administration of high doses of TNF-α was required to sustain the plasma TNF-α level for observing significant antitumor effects because of its short plasma half-life, and TNF-α as a systemic antitumor agent was found to have unexpected toxic side-effects in phase I studies [6]. This severe toxicity of TNF-α prevented the administration of dosages required for antitumor activity observed in preclinical studies. Thus, TNF-α is administered only into the tumor or the artery controlling cancer in the current cancer chemotherapy [19–22]. The antitumor effects of TNF-α result from not only its direct cytotoxic action against various tumor cells, but also specific damage to the tumor vessels. Additionally, in the process of bleeding necrosis in the tumor vessels, the vascular permeability of the tumor vessels is selectively increased, promoting the transport of macromolecules from blood to the tumor tissue. On the other hand, the increase in blood-residency would lead to a decrease in the distribution of TNF-α in the liver and spleen, which are the major sources of the unfavorable side-effects. Thus, an improvement in blood residency of TNF-α may expand its antitumor therapeutic window. Recently, we prepared phage libraries expressing mTNF-αs, in which all the lysine residues were replaced with other amino acids [16]. A fully bioactive lysine-deficient mutant TNF-α [mTNF-α-Lys(−)] was obtained, despite reports that some lysine residues were essential for its bioactivity. mTNF-α-Lys(−) was site-specifically mono-PEGylated at its N-terminus with LPEG$_{5K}$. This sp-LPEG$_{5K}$-mTNF-α-Lys(−) showed a longer plasma half-life than wTNF-α, resulting in an improvement of in vivo antitumor potency compared to wTNF-α. In this study, to obtain the fundamental information for designing a more useful sp-PEG-mTNF-α-Lys(−) as a systemic antitumor agent, we attempted to predict the structure of mTNF-α-Lys(−) through molecular modeling and assess the various properties of sp-PEG-mTNF-α-Lys(−)s.

A matter of great interest was why mTNF-α-Lys(−) and wTNF-α had comparable in vitro bioactivity after the substitution of all the lysine residues. Site-directed mutagenesis analysis of TNF-α suggested that among six lysine residues, Lys65 and Lys90 were involved in interaction with its receptor, in particular. Fig. 4 showed the model complex between wTNF-α and TNF-receptor I (TNF-RI). In the wTNF-α structure, Lys65 was predicted to repel against Lys78 in TNF-RI. Lys90 forms a hydrogen bond with Glu135 and this interaction is likely to stabilize the loop structure containing residues 84–89, which involved in the receptor binding in the model. In mTNF-α-Lys(−), Lys65 was replaced by Ser65. We considered that the substitutions of Lys65 with a small amino acid, such as Ser65 in mTNF-α-Lys(−), would enable these proteins to bind receptors because of loss of interference between Lys65 in TNF-α and Lys78 in TNF-RI. However, in mTNF-α-Lys(−), Pro90 is likely to be unable to form hydrogen bond with Glu135 and the loop structure became unstable. Therefore, mTNF-Lys(−) had comparable in vitro bioactivity to wTNF-α.

As mentioned above, Lys65, and Lys90 in wTNF-α were reported to play important roles at least for the expression of their bioactivities. Thus, the introduction of PEG to these lysine residues should have caused a significant loss of its bioactivity. In fact, the in vitro bioactivity and receptor-affinity of ran-LPEG$_{5K}$-wTNF-α were markedly lower than those of wTNF-α (Fig. 2 and Table 1). Additionally, we previously found that random PEGylation of wTNF-α with larger Mw of LPEG resulted in much lower bioactivity of ran-LPEG-wTNF-. Previously we showed that ran-LPEG$_{5K}$-wTNF-α was composed of positional isomers with a LPEG$_{5K}$ molecule at various lysine residues, and such isomers could have distinct remaining bioactivity compared with wTNF-α [16]. In most cases, these drawbacks of random PEGylation of bioactive proteins have prevented the clinical application of the PEGylated ones. In contrast, the N-terminus of TNF-α was found to be unimportant for its function, because a deletion mutant lacking eight residues at the N-terminus retained its full bioactivity [16]. As a result, the remaining in vitro bioactivity of each sp-PEG-mTNF-α-Lys(−) was more than 60% compared with both wTNF-α and mTNF-α-Lys(−). In particular, sp-BPEG$_{40K}$-mTNF-α-Lys(−) had a 20-fold higher bioactivity than ran-LPEG$_{5K}$-wTNF-α.

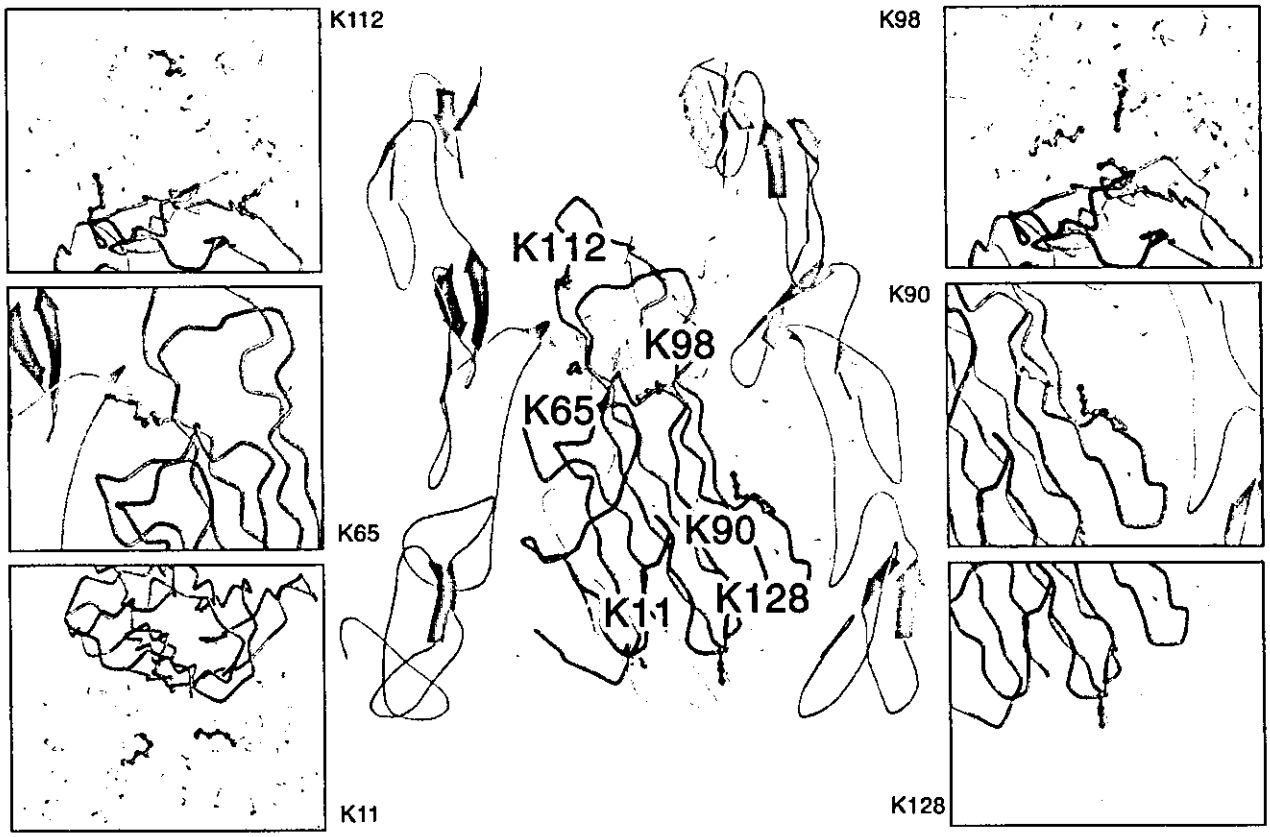

Fig. 4. Ribbon diagrams of the complex model between wTNF-α and TNF-RI. This model is based on crystal structures of wTNF-α trimer (PDB code:1TNF) [23] and TNF-β/TNF-RI complex (PDB code:1TNR) [24]. The images were drawn using Bobscript [25] and Raster3D [26]. Each subunit of the wTNF-α is shown in blue, green, and yellow. TNF-RI is shown in magenta.

Additionally, in the case of mTNF-α-Lys(−), both activated LPEG and BPEG were introduced only into the N-terminal amino group. Thus, the obtained sp-PEG-mTNF-α-Lys(−)s were molecularly uniform. These results strongly indicated the usefulness of our site-specific PEGylation system for lysine-deficient mutant proteins with full bioactivity, which could be created by the phage display technique. The N-terminus of many cytokines, such as interleukin-2, is not important for its function. Thus, at present, the creation of lysine-deficient mutant cytokines and its site-specific PEGylation are being carried out for promotion of cytokine therapies.

$BPEG_{10K}$ and $BPEG_{40K}$ have two LPEG molecules with Mw of 5 and 20 kDa, respectively. Thus, the Ms of PEGylated molecules efficiently increases by PEGylation with BPEG rather than that with LPEG, and BPEG may be more suitable than LPEG. In fact, the remaining in vitro bioactivities and receptor-affinity of sp-$BPEG_{10K}$-mTNF-α-Lys(−) and sp-$BPEG_{40K}$-mTNF-α-Lys(−) were similar to those of $LPEG_{5K}$-mTNF-α-Lys(−) and sp-$LPEG_{20K}$-mTNF-α-Lys(−), respectively (Fig. 2 and Table 1). Additionally, the blood-residency of sp-PEG-mTNF-α-Lys(−) enhanced with an increase in its Ms, irrespective of the molecular shape of PEG (data not shown). In case of PEGylation of TNF-α, sp-$BPEG_{40K}$-mTNF-α-Lys(−), whose remaining in vitro bioactivity was about 65% compared with mTNF-α-Lys(−), did not show any antitumor response (Fig. 3 and Table 1). In vivo antitumor potency of other sp-PEG-mTNF-α-Lys(−)s was higher than that of mTNF-α-Lys(−). A matter of great interest is the difference in in vivo activity of sp-PEG-mTNF-α-Lys(−)s. This difference may be partly accounted for by tissue transport. sp-$BPEG_{40K}$-mTNF-α-Lys(−) might be hard to transport to the tumor tissue than other sp-PEG-mTNF-α-Lys(−)s, because of higher Ms. Thus, we believed that sp-$BPEG_{10K}$-mTNF-α-Lys(−) and sp-$LPEG_{20K}$-mTNF-α-Lys(−) are the most optimal modified products. However, detailed studies on the pharmacokinetics of sp-PEG-mTNF-α-Lys(−)s are necessary to clarify our speculation, and these are currently under investigation.

In a previous study, we showed that the conjugation of TNF-α with polyvinylpyrrolidone (PVP) effectively increased its antitumor effects as compared to that with PEG because PVP has superior character than PEG for the purpose of prolonging plasma half-life and increasing stability [13]. Therefore, we consider that the application of PVP to site-specific bioconjugation of mTNF-α-Lys(−) would be useful as a more potent antitumor therapeutic agent. Our results indicated that it is necessary to optimize the condition of PEGylation to selectively enhance the desirable therapeutic effects, taking into account their mechanism of action. Our results provide the information necessary to design a PEGylated protein, optimally suitable for therapeutic use.

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Selective Enhancer of Tumor Vascular Permeability for Optimization of Cancer Chemotherapy

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Clinical approach using tumor necrosis factor-alpha (TNF-α) as selective destruction against tumor endothelial cells and selective enhancer of tumor vascular permeability for effective accumulation of antitumor chemotherapeutic agents has attracted attention. However, the clinical application of TNF-α as a systemic antitumor agent has been limited because of toxic side-effects. To systemically use TNF-α as an antitumor agent and the selective enhancer of tumor vascular permeability, we assessed the usefulness of PEGylated TNF-α (PEG-TNF-α). PEG-TNF-α at a dose of 1000 JRU showed marked hemorrhagic necrosis in S-180 tumors without side-effects due to selective destruction of tumor vasculature, whereas wild-type TNF-α at a dose of 10000 JRU showed a little hemorrhagic necrosis with severe side-effects. PEG-TNF-α induced the enhancement of tumor vascular permeability. The permeability was increased at 1 h, after an i.v. injection of PEG-TNF-α and returned to the basal level at 2 h. In addition, high molecular weight of PEG (molecular weight; 500K) accumulated in tumor tissue as well as low molecular weight of PEG (molecular weight; 12K). On the other hand, PEG-TNF-α didn't affect the permeability of normal tissue and inflammation site. This data suggested that PEG-TNF-α was useful agent as selective enhancer of tumor vascular permeability with safe.

Key words tumor necrosis factor-alpha; permeability; tumor endothelial cell; chemotherapeuctic drug

With the development of tumor biology, molecular biology, and combinatorial chemistry, many therapeutic agents have been developed for tumors. However, the clinical application of antitumor agents such as cytokines, antibodies and chemotherapeutic drugs has failed because of poor accumulation in the tumor tissue.[1,2] Heterogeneous tumor perfusion, vascular permeability, and increased interstitial pressure restricted the penetration of therapeutic agents into tumor tissue from circulation.[1,2]

It has been suggested that tumor necrosis factor-alpha (TNF-α) can overcome these problems. TNF-α was identified as a cytokine that specifically injures tumors and has been highlighted as a potent anti-tumor agent.[3] Furthermore, TNF-α destroyed the tumor vasculature selectively and enhanced the tumor vascular permeability.[4] Regional isolated limb perfusion using TNF-α in combination with melphalan or doxorubicin showed greater therapeutic effects on soft-tissue sarcoma or melanoma than chemotherapeutic drugs alone.[5] It is believed that the synergistic effect between TNF-α and chemotherapeutic drugs results from TNF-α-induced destruction of tumor vasculature and enhancement of tumor vasculature permeability. However, because of its high instability and pleiotropic action *in vivo*, attempts to use TNF-α as a systemic anticancer agent and an enhancer of tumor vascular permeability in humans, failed due to severe systemic side effects such as fever and decreased blood pressure as seen in case of an endotoxin-like shock, before therapeutic doses could be administered.[6]

To overcome these drawbacks and apply TNF-α as a systemic administrator, we have attempted to conjugate TNF-α with polyethylene glycol (PEG) and other water-soluble polymeric modifiers.[7—10] We showed that the antitumor effects of PEG-TNF-α, obtained by PEGylation with a molecular weight of 5000, was enhanced to 100 times that of unmodified TNF-α, without increasing their toxic side effects.[7,8] In this study, we examined the usefulness of PEG-TNF-α as a selective enhancer of tumor vascular permeability. We showed that PEG-TNF-α enhanced the permeability of tumor vasculature without having any effect on the permeability of normal tissue. We considered that PEG-TNF-α would be used with chemotherapeutic drugs for tumor therapy.

MATERIALS AND METHODS

Preparation of ^{125}I-Labeled Polymers Radiolabeled polymeric modifiers were prepared by the chloramine-T method. PEGs (average molecular weight: 12000, 50000, 70000, 500000) (purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 1,4-dioxan were reacted with *N,N'*-carbonyldiimidazole for 6 h at room temperature. After dialysis in water, the activated polymers were reacted with a 2-fold molar excess of thyramine hydrochloride for 48 h at 4 °C. These reaction mixtures were also dialyzed in water and lyophilized. The polymer–thyramine conjugates, dissolved in a 0.4 M sodium phosphate buffer (2.5 mg/ml) and Na^{125}I (100 mCi/ml), were mixed in a microcentrifuge tube on ice. The labeling reaction was started by the addition of 3.8 mM chloramine T. After iodination, the reaction was stopped by the addition of 2.5 mM sodium pyrosulfate. ^{125}I-Labeled polymer was purified by Gel Filtration Chromatography on the Econo-Pac® 10 DG column.

***In Vivo* Behavior of Polymers** All experimental protocols for animal studies were in accordance with the *Guide for Laboratory Animal Facilities and Care* (NIH publication 85-23, rev 1985). These protocols have been approved by the committee of the Pharmaceutical School, Osaka University, Japan. PEG-TNF-α, in which 56% of the lysine amino groups of natural human TNF-α (Hayashibara Biological Laboratories Inc., Okayama, Japan) were coupled with PEG

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and prepared as described.[7] S-180 cells were implanted intradermally (5×10^5 cells/200 μl/site) in 5-week-old female BALB/c mice. On day 7, when the tumor diameter reached 7 mm, PEG-TNF-α [500 Japan Reference Unit (JRU)/mouse] was injected i.v. into the mice. At 1, 1.5, 2, 3, 4, and 6 h later, various molecular weight of ^{125}I-Labeled PEG (1×10^6 cpm/200 μl) were administered i.v. Thirty minutes after the injection of ^{125}I-Labeled PEG, the mice were killed by abdominal arterial exsanguination. Then, the liver, kidney, spleen, heart, lungs, brain, inflammatory sites in which cotton had been implanted, skin and tumor were recovered and their radioactivity was measured by a γ-counter. The vascular permeability (VP) ratio was calculated according to the following equation.

$$\text{VP ratio} = \frac{\text{the radioactivity of tissue after the administration of PEG-TNF-}\alpha}{\text{the radioactivity of tissue without the administration of PEG-TNF-}\alpha}$$

RESULTS AND DISCUSSION

Recently, TNF-α has been clinically applied to loco-regional combination therapy with Melphalan, and this therapy showed a marked antitumor effect for patients in transit melanoma metastases.[11] This clinical approach, using TNF-α as a selective destruction agent against tumor endothelial cells and as a selective enhancer of tumor vascular permeability for effective accumulation of antitumor chemotherapeutic agents, is presently an attractive topics in the study of the optimization of cancer chemotherapy. Furthermore, it has also been approved by the European Agency for the Evaluation of Medicinal Products.[12] However, systemic administration of TNF-α was restricted due to its side effects. To use TNF-α for various therapeutic applications, we have created PEG-TNF-α with superior both in antitumor effectiveness and safety.

PEG-TNF-α at a dose of 1000 JRU showed marked hemorrhagic necrosis in S-180 tumors by a single i.v. injection without side-effects (Fig. 1). In mice treated with native TNF-α, a little hemorrhagic necrosis was caused at 10000 JRU. However, one of seven mice treated with native TNF-α at this dose died within 24 h, and the remaining mice developed piloerection and tissue inflammation (*e.g.*, erythma) and showed a decrease in body weight. Therefore, we considered that native TNF-α was not appropriate for examining the biological function and the application of therapy by systemic administration.

Figure 2 shows the vascular permeability (VP) induced in various tissues using PEG-TNF-α. The VP of tumor blood vessels formed in S-180 tumors was increased at 1—2 h, after an i.v. injection of PEG-TNF-α. At 3 h after the injection of PEG-TNF-α, the VP returned to the control-basal level, and it was lower than the control-basal level at 3 h. Furthermore, PEG with high molecular weight (molecular weight=500000) could accumulate in the tumor tissue as well as PEG with low molecular weight (molecular weight=12000). On the other hand, the permeability of the vessels in normal tissues, such as the brain, lung, liver, spleen, kidney, and skin, or inflammatory sites in which cotton had been implanted, changed slightly by i.v. injection of PEG-TNF-α.

Many researchers reported that TNF-α induces change of endothelial cytoskeletal actin and formation of intercellular gaps with increased permeability to macromolecules.[13,14] In addition, we previously reported that endothelial cells cultured in conditioned medium prepared from tumor cells, converted normal endothelial cells to have various character similar to tumor endothelial cells, and these cells had highly sensitivity to TNF-α.[15,16] Therefore, the increased permeability of the newly formed vessels in the system at 1—2 h after the administration of PEG-TNF-α would result from the increasing of the permeability of tumor endothelial cells and the cytotoxicity of PEG-TNF-α on tumor vascular endothelial cells. On the other hand, TNF-α is known to have procoagulant effects on tumor neovasculature, by causing fibrin deposition and localized thrombosis, which, in turn, leads to ischaemic necrosis of tumors.[17] Therefore, normalized and decreased permeability at 3 h would reflect the cessation

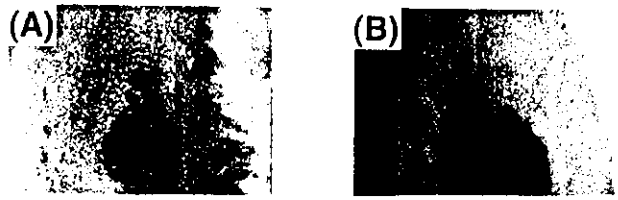

Fig. 1. Hemorrhagic Necrosis Effects of PEG-TNF-α

At 7 d after the tumor inoculation, mice were treated with i.v. injection of the (A) native TNF-α: 10000 JRU/mouse, (B) PEG-TNF-α: 1000 JRU/mouse. Tumor hemorrhagic necrosis was observed 24 h after the injection by macroscopic observation.

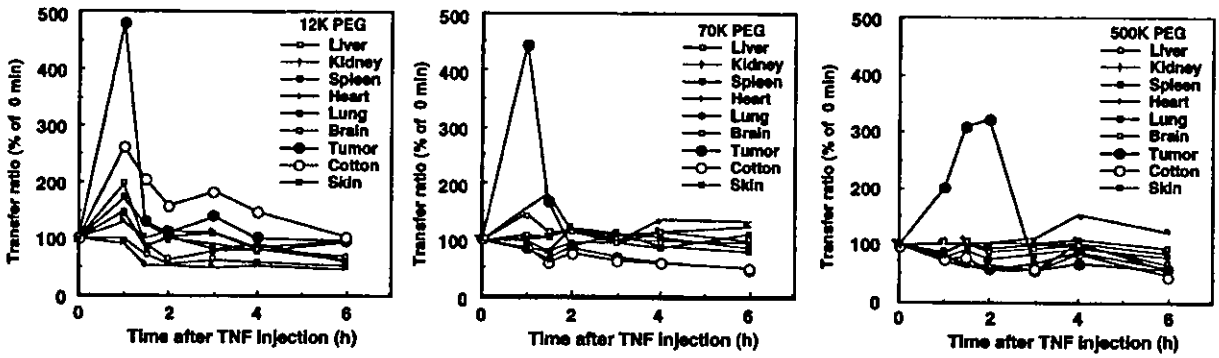

Fig. 2. Vascular Permeability in Various Tissues with PEG-TNF-α Treatment

Mice bearing S-180 tumor were killed at the indicated times after i.v. injection of PEG-TNF-α (500 JRU/ml). Thirty minutes before killing, each mouse was pulsed by an i.v. injection of ^{125}I-labeled PEG at a dose of 400000 cpm. Control levels without pre-injection of PEG-TNF-α are shown for time 0.

of the blood circulation because of the adsorption of fibrin-like substances on the luminal surface of the tumor vascular endothelial cells.

A matter of interest was why PEG with high molecular weight (molecular weight=500000) showed highest VP at 2 h when PEG with low molecular weight (molecular weight=12000 or 70000) could not accumulate in the tumor. The degree of accumulation into the tumor was decided by the balance between the penetration into the tumor tissue from the circulation and the leak from the tumor. Therefore, at 1 h after the administration of PEG-TNF-α, PEG with low molecular weight might accumulate in the tumor easily and accumulate in the tumor without leak. However, PEG with high molecular weight could not penetrate into the tumor. At 2 h after the administration of PEG-TNF-α, PEG with low molecular weight might penetrate into the tumor easily and leak from the tumor into the circulation. On the other hand, PEG with high molecular weight could penetrate into the tumor and accumulate in the tumor without leak.

Recently, many bioactive proteins such as immunotoxin, antibody-toxin conjugates, and cytokine has been attracted a highly expectation for tumor therapy. However, there has been no significant therapeutic response to solid tumors due to insufficient tumor accumulation. Therefore we consider that PEG-TNF-α would overcome these drawbacks. We are now examining the usefulness of PEG-TNF-α for combination therapy using chemotherapeutic drugs or immunotoxin. Thus PEG-TNF-α will open the new way to combination therapy with antitumor agents.

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Recent Progress on Tumor Missile Therapy and Tumor Vascular Targeting Therapy as a New Approach

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Abstract: Tumor targeting therapy, that is "Missile therapy", using a complex composed of a tumor suppressive drug and a whole antibody against tumor cells, is expected to become an attractive chemotherapy strategy. However, clinically convincing results have not yet been obtained mainly due to poor transport from the circulation to tumor tissue and marked toxicity. Recently, recombinant immunotoxins, composed of an Fv fragment of an antibody to a tumor-related antigen fused to various truncated toxins have been developed to overcome the distribution of immunotoxins in tumors. These recombinant immunotoxins have shown encouraging clinical results for some hematopoietic malignancies. However, there were no significant anti-tumor responses to many tumors, especially solid tumors, probably due to their rapid clearance from the circulation and their immunogenicity and antigenicity. More recently, PEGylation of recombinant immunotoxins has been attempted to overcome these drawbacks. It was found that PEGylation of recombinant immunotoxins improves their effectiveness. We discuss the recent progress in tumor missile therapy. In contrast to others, we developed "Missile therapy against tumor blood vessels" by using specific monoclonal antibodies against tumor endothelial cells rather than actual tumor cells. The complex between antibodies to tumor vascular endothelial cells and anti-tumor drugs can freely access the target cells without concern for their vascular permeability. These preparations have exhibited excellent anti-tumor effects for solid tumors. In this review, we also discuss this vascular targeting therapy as an attractive new strategy for tumor chemotherapy.

Keywords: Immunotoxin, PEGylation, Tumor vascular targeting, Antibody, Immunoconjugate, Tumor necrosis factor-alpha, Interleukin-6, Tumor endothelial cells.

1. INTRODUCTION

In modern tumor therapy, the paucity of efficient and target-specific anti-tumor drugs that are without serious side effects is a considerable problem. Therefore, drug delivery systems (DDS), such as immunoconjugates and immunotoxins, which are composed of anti-tumor agents (anti-tumor antibiotics and toxins) and monoclonal antibodies against a tumor-associated antigen (TAA), are being studied by many investigators [1-6]. However, this tumor missile therapy (Targeting Therapy) has not yielded satisfactory results [6,7], except in some limited cases, for the following reasons: (1) when a whole monoclonal antibody is used as a missile molecule, the complex of an antibody and an anti-tumor agent (immunoconjugate/ immunotoxin) has a very large molecular weight (over 150,000), resulting in poor selective transfer to the tumor tissue [8,9]; (2) the preparation of immunoconjugates or immunotoxins requires complex processes and it is not easy to produce them in adequate amounts for clinical use; (3) cross-linking monoclonal antibodies to anti-tumor agents often reduces the antibody titer or anti-tumor cytotoxic activity; and (4) monoclonal antibodies or toxins used as anti-tumor agents, which generally have stronger cytotoxicity to tumor cells than anti-tumor antibiotics, sometimes acquire antigenicity or immunogenicity, which makes their repeated administration difficult and elevates the risk of anaphylaxis. This reduces their anti-tumor effects while inducing unexpected adverse reactions. Recently, Pastan *et al.* have partially resolved these limitations [10,11]. They attempted to improve drug transfer to tumor tissue by using the variable region (Fv region) of antibodies as missile molecules. They succeeded in the large-scale production of recombinant immunotoxins that exhibit completely preserved antibody titers and toxin activity. Genetic engineering, using a fusion protein composed of the Fv portion of an antibody (that recognizes the tumor-specific antigens) and toxins, was used for this purpose. Excellent preclinical results of these recombinant immunotoxins *in vitro* and *in vivo* have led to the initiation of several clinical trials [12-14]. However, some immunotoxins have problems with stability, immunogenicity and lack of specificity. Thus, the elimination of these problems will elucidate a new approach to immunotoxin therapy.

As mentioned above, recombinant immunotoxins have been showing excellent anti-tumor responses to hematopoietic malignancies in Phase I trials but have not shown significant clinical effects to solid tumors (e.g. colon). This is probably due to their short plasma half-lives and

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other factors. Angiogenesis is a prominent feature of many physiological and pathological processes, including wound healing, luteinization and tumor growth [15,16]. Tumor angiogenesis is mediated by substances produced by tumor cells, and many previous studies have demonstrated that tumor growth is dependent upon it. Angiogenesis is also critical for tumor growth, since tumor vasculature not only supplies some nutrients but also mediates waste removal. Thus, selective inhibition of tumor angiogenesis and/or destruction of the tumor vascular -network seemed to be a more attractive approach for controlling neoplasms than direct anti-tumor therapies [17-20]. Tumor cells are heterogeneous [21,22]: therefore, a common antibody applicable to a wide range of tumor types does not exist. Conversely, almost all tumor tissue blood vessels exhibit various common features, such as enhanced permeability regardless of the tumor type or animal species (however, a whole antibody of molecular weight 150 kDa cannot rapidly access the solid tumor tissue from the circulation), suggesting the expression of common specific molecules in tumor tissue vascular endothelial cells (TEC), typified by the VEGF receptor. In addition, targeted TEC are directly exposed to blood, so their transfer from blood to the tumor tissue need not be considered.

In this review, we first, show the recent progress in tumor missile therapy using recombinant immunotoxins. We also summarize the essential technologies for ensuring the efficacy and safety of recombinant immunotoxins. Finally, we describe the usefulness of tumor vascular targeting therapy as a new approach to treatment.

2. RECOMBINANT IMMUNOTOXINS TO TUMOR CELLS

Recombinant immunotoxins are chimeric proteins in which a truncated toxin that serves as the cytotoxic moiety is fused to the Fv fragment of an antibody that serves as the targeting moiety. Pastan *et al.* produced a recombinant immunotoxin composed of an Fv fragment of an antibody to a tumor-related antigen fused to a 38-kDa mutant form of Pseudomonas exotoxin A (PE) [11.23-25]. Using several antibodies to various tumor-related antigens, they made recombinant immunotoxins by deleting the cell-binding domain of PE and replacing it with the Fv portion of an antibody. Some of these recombinant immunotoxins have recently been evaluated in Phase I trials. All the recombinant immunotoxins have been shown to cause complete regression of human tumor xenografts in nude mice and are relatively well tolerated by mice and monkeys.

For example, anti-Tac(Fv)-PE38 (LMB2, anti-CD25 recombinant immunotoxin), containing a single-chain Fv fragment of the anti-human Tac monoclonal antibody to the IL-2 receptor alpha subunit fused to a fragment of Pseudomonas exotoxin (PE38) with the translocation and ADP-ribosylation domains of PE, was recently found to be the first recombinant immunotoxin to induce major clinical responses in tumors [12,26]. Anti-Tac(Fv)-PE38 was administered to 35 patients with CD25+ hematologic malignancies, who failed standard and salvage therapies. As a result, one patient with hairy cell leukemia (HCL) had a complete remission [12], on going at 16 months, and seven partial responses were observed in HCL (n=3), cutaneous T-cell lymphoma (n=1), chronic lymphocytic leukemia (n=1), Hodgkin's disease (n=1), and adult T-cell leukemia (n=1).

However, anti-Tac(Fv)-PE38 exhibits, which limits the amount of immunotoxin that can be administered. These toxic were probably due to its non-specific distribution from the circulation to various normal organs, because it was found that the dose-limiting toxicity of anti-Tac(Fv)-PE38 in the Phase I trials was most often due to damage of liver cells that do not express IL-2 receptors. In addition, human anti-mouse antibodies and anti-PE antibodies were elevated in some patients treated with anti-Tac(Fv)-PE38, and this immunogenicity of recombinant immunotoxins may reduce their therapeutic potency. These problems may occur with other recombinant immunotoxins as well. Thus, if these side effects can be avoided, recombinant immunotoxins will show improved responses in human trials.

3. IMPROVEMENT OF THE NON-SPECIFIC TOXICITY OF RECOMBINANT IMMUNOTOXINS BY MUTATIONS IN THE FRAMEWORK REGIONS OF THE FV REGION

The toxic side-effects of recombinant immunotoxins are of two types. One type of toxicity results from the specific targeting of normal cells that express the same antigen as the tumor cells. Thus, Pastan *et al.* have been identifying more specific antigens present on prostate and ovarian tumor by using the latest technique in molecular biology such as DNA chip technology, phage display and DNA immunization as novel preparative methods for Fv polypeptides against tumor cells [5,27,28]. The other type of toxicity is non-specific and is usually characterized by damage to liver cells, although other toxic effects may also occur. Recombinant immunotoxins are foreign proteins for humans and mice; they may distribute to the reticuloendothelial system (RES), such as the Kupffer cells in the liver. In addition, these recombinant immunotoxins (Mw: 65,000) have a lower molecular weight than conventional antibody-toxin conjugates (Mw: 195,000) leading to a shorter survival in circulation and increased distribution to various normal tissues such as kidney and liver. For example, anti-Tac(Fv)-PE38 showed marked anti-tumor effects in leukemias and lymphomas in a Phase I trials. But it was found that one of the reasons for the limitation of dose escalation of anti-Tac(Fv)-PE38 was liver toxicity. If this liver toxicity of recombinant immunotoxins is overcome, the clinical results will be improved. Pastan *et al.* noted that the Fv of anti-Tac has an isoelectric point (pI) of 10.2 [29,30]. They hypothesize that the overall positive charge on the Fv portion of anti-Tac(Fv)-PE38 contributes to the non-specific binding to liver cells and results in dose-limiting liver toxicity. They found that lowering the pI of the Fv of anti-Tac, from 10.2 to 6.8 by selective mutation of surface residues, causes a 3-fold decrease in animal toxicity and hepatic necrosis in mouse models. This change in pI did not significantly alter the CD25 binding affinity, the cytotoxic activity towards target cells, or anti-tumor activity *in vivo*. These mutations in the framework regions of the Fv, which lower the pI, were found to improve the toxicity of other recombinant immunotoxins, such as SS1(dsFv)-PE38 targeting ovarian tumor and B3(dsFv)-PE38 targeting colon and breast tumors. If this

decreased toxicity also occurs in humans, it should markedly enhance the clinical effects.

4. PEGYLATION OF BIOACTIVE PROTEINS

As mentioned above, recombinant immunotoxins often exhibit immunogenicity and antigenicity, and this reduces their therapeutic usefulness. In addition, it was found that the plasma half-lives of these recombinant immunotoxins were unexpectedly short. Preclinical studies have shown that anti-tumor activity in hematopoietic malignancies is enhanced if recombinant immunotoxins survive longer in the circulation [31]. One of the most common ways to increase the blood-residency of proteins is to modify them with polyethylene glycol (PEG) [32-40] (Fig. 1). Many studies have reported that chemical modification of proteins with PEG (PEGylation) increases the molecular size and steric hindrance of the protein [41-44]. This results in improved plasma half-life and proteolytic-stability, and decreased immunogenicity and hepatic uptake. PEGylation of bioactive proteins decreases their renal excretion rate due to the increased molecular size. In addition, since the PEG chain covers the protein surface, attack from proteinases is blocked by steric hindrance, resulting in prolongation of the *in vivo* half-life. Similar steric hindrances cause a decrease in antigenicity and immunogenicity, resulting in the prolongation of *in vivo* clearance and stability. Due to these comprehensive effects of PEGylation, the doses of bioactive proteins can be decreased, resulting in reduced toxic side effects. Thus, the optimal molecular designs of proteodrugs are considered to be developed by PEGylation. For example, PEGylation of interleukin-2 has been reported to increase its anti-tumor potency *in vivo* and PEGylation of an F (ab') 2, derived from monoclonal antibody A7 has improved its localization to tumors. Recently, we also attempted the PEGylation of some bioactive proteins, such as tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) and superoxide dismutase (SOD) to improve their therapeutic potencies. In this section, we show, using TNF-alpha and IL-6 as examples, the possibility that PEGylation of bioactive proteins, such as recombinant immunotoxins, overcomes their drawbacks, allowing their clinical application.

The recent marked advances in molecular biology and genetic engineering have enabled the large-scale production of a number of bioactive proteins, such as cytokines and recombinant immunotoxins. Attempts at applying these recombinant bioactive proteins to the treatment of intractable diseases such as tumors have been receiving close attention. However, since almost all of these recombinant bioactive proteins are quite unstable *in vivo*, their clinical use as a therapeutic agent requires frequent administration and high doses, which can impair homeostasis and cause severe adverse effects [46]. Furthermore, since cytokines such as TNF-alpha and IL-6, have diverse biological actions on various tissues, it is not easy to selectively obtain some particular favorable action (therapeutic effects) among their diverse actions and minimize side effects. These disadvantages markedly limit the clinical use of cytokines [46-48]. These problems concerning cytokines also apply to many other bioactive proteins typified by recombinant immunotoxins, whose large-scale production has become possible in recent years. Therefore, it is essential not only to develop a DDS that can overcome their instability *in vivo* but also to establish DDS technology that selectively exerts some particular beneficial action among their diverse

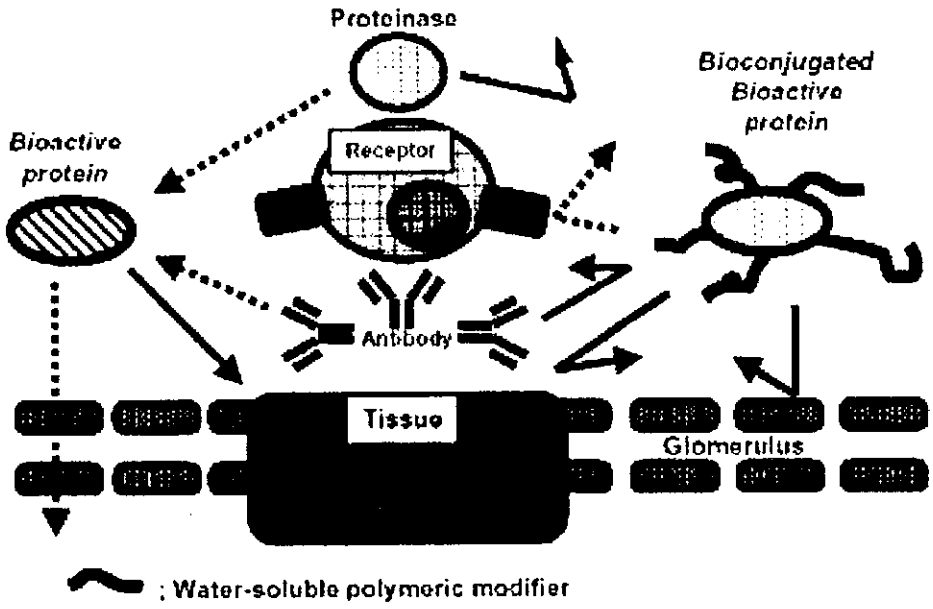

Fig. (1). Characteristics of hybrid bioactive proteins.
Hybrid bioactive proteins with water-soluble polymeric modifiers increase their molecular size and steric hindrance, resulting in augmented plasma half-lives and stability. For example, PEGylation enables the therapeutics dose and frequency to be decreased.

biological actions, i.e., a technology, which allows the exertion of only the targeted therapeutic action, without being accompanied by side effects. Keeping these points in mind, we attempted the bioconjugation of bioactive proteins using water-soluble polymeric modifiers such as PEG, to improve the stability of these proteins *in vivo*, and to achieve a more selective exertion of their therapeutic actions.

The behavior of bioconjugated bioactive proteins is expected to be greatly affected by the features of the polymeric modifiers, which cover the surface of these proteins. Therefore, it is necessary to first identify the particular polymeric modifiers, which provide desirable behavioral characteristics *in vivo* to individual bioactive proteins, taking into consideration the mechanisms by which these proteins exert their favorable actions. To prepare an index for selecting optimum polymeric modifiers, we evaluated the behaviors and pharmacokinetics of various polymeric modifiers *in vivo* and their interactions with vascular endothelial cells *in vitro*. This evaluation revealed that polymeric modifiers that possess charged functional groups or hydrophobic residues selectively accumulate in certain tissues such as kidney, liver and spleen, depending on the type and density of the functional groups. Additionally, non-ionic water-soluble polymers typified by PEG do not show a marked transfer to tissue and are suitable for the use in bioconjugation with proteins to improve their retention in blood.

When IL-6 is utilized therapeutically to promote the formation of platelets, its targets are the megakaryocytes [49,50]. It is known that megakaryocytes express large amounts of high affinity IL-6 receptors (IL-6 receptor-gp130 complex) [51] and that they are abundant in the pulmonary vascular lumens and on the outer surface of marrow veins [49]. Therefore, if IL-6 is modified to remain longer in circulation and thus smaller doses will be required for therapeutic use, then it will be possible to make the distribution and receptor affinity of IL-6 *in vivo* such that a selective and efficient action of IL-6 on megakaryocytes can be achieved. Furthermore, since the transfer of IL-6 into the liver and spleen causes adverse reactions [52], the improved retention of IL-6 in the blood and the resultant decrease in the transfer and accumulation of IL-6 to these tissues is expected to reduce the side effects of IL-6 therapy. Thus, we attempted the bioconjugation of IL-6 with PEG (PEGylation) to increase the activity of IL-6 in the promotion of platelet production and reduce its side effects. When IL-6 was subjected to PEGylation under optimum conditions that were selected by considering the relationships between specific activity, degree of PEG-modification, molecular size, etc., the resultant PEG-modified IL-6 (MPEG-IL-6) had a plasma half-life over 100 times greater than that of native IL-6. MPEG-IL-6 showed more than 500 times the thrombopoietic potency of native IL-6 (Fig. 2). Furthermore, strong adverse reactions such as fever, IgG production and acute protein production observed following the administration of native IL-6 were seldom seen after the administration of MPEG-IL-6. Separation of therapeutically favorable targeted actions from side effects, using PEGylation, has been successful not only for IL-6 but also for TNF-alpha. Our study demonstrates that the PEGylation of TNF-alpha, under optimum conditions, remarkably improved the stability of

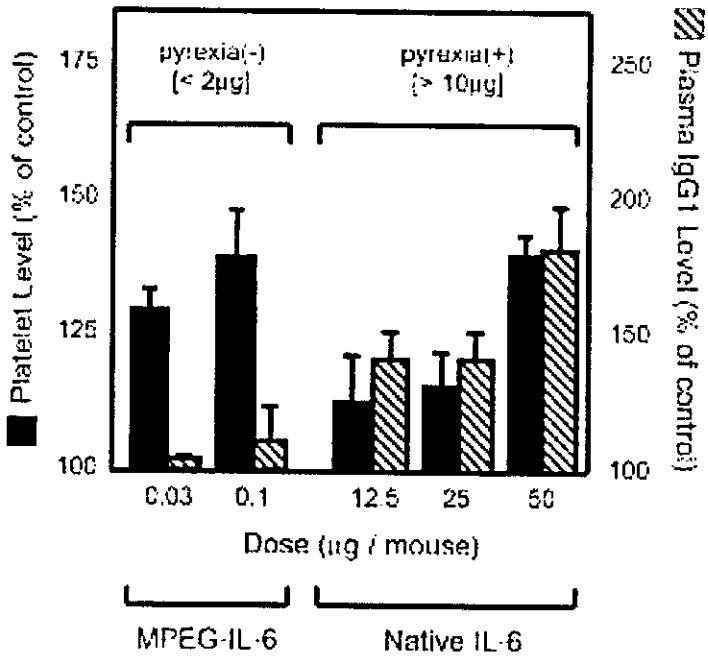

Fig. (2). Effects of PEG-IL-6 on platelet production and serum IgG1 level.
Blood samples of mice on day 9 after injection were used every 2 days. Serum IgG1 concentration was measured by ELISA. (Mean ± SE, N=4.)

TNF-alpha in blood and its transfer to tumor tissue resulting in a greater therapeutic efficacy of TNF-alpha (about a 100-fold increase in anti-tumor activity, compared to native TNF-alpha), while reducing the adverse reactions. It was found that PEGylation allows the exertion of selected favorable actions of cytokines via the following mechanisms: (1) improved stability *in vivo* reduces the dosage and thus the blood level of cytokines, making it possible for a given cytokine to exert its selected actions on the basis of the differences in the affinity of the cytokine for various receptors; and (2) regulation of the behavioral characteristics (blood retention and tissue transfer) of a given cytokine enables the cytokine to exert selected actions, depending on the differences in its distribution among various tissues. We have thus succeeded in making cytokines useful as therapeutic agents by improving their stability *in vivo* and increasing their selected favorable actions (anti-tumor activity in the case of TNF-alpha and the promotion of platelet production in the case of IL-6). These results suggest that optimal PEGylation will also be useful in improving the clinical problems of recombinant immunotoxins.

5. SITE-SPECIFIC PEGYLATION OF RECOMBINANT IMMUNOTOXINS AND THE IMPROVEMENT OF THEIR PLASMA HALF-LIVES, STABILITY AND IMMUNOGENICITY.

Pastan *et al.* reported that a PEGylated chimeric toxin composed of transforming growth factor-alpha and PE, showed an improved blood-residency time and a decrease in immunogenicity resulting in enhanced *in vivo* anti-tumor potency and reduced *in vivo* toxicity [58]. However, PEGylation was accompanied by a significant loss of specific cytotoxic activity of PE. Unlike PEGylation of enzymes, which act on small substrates, the PEGylation of recombinant immunotoxins may cause a decrease in activity due to loss of antigen-binding, ADP-ribosylation, or the ability to translocate to the cytosol, which are based on macromolecular interactions that are easily sterically hindered by the attached PEG. We also obtained similar results. The specific activity of PEGylated TNF-alpha decreased with the PEG modification rate (degree of PEG-modification). Lys11 is considered to play an important role in the development of TNF-alpha activity. Thus, a decrease in the specific activity may be partly due to the modification of the lysine residues involved in the development of TNF-alpha activity. Additionally, the bioactivity of PEGylated TNF-alpha decreased with an increase in the molecular weight of the attached PEG. Our other studies on PEGylated IL-6 and leukemia inhibitory factor (LIF) yielded similar results. In our previous study on PEGylated SOD, SOD-substrate complex formation was possible without steric hindrance of the PEG attached to SOD because of the low molecular weight of the substrate (superoxide anion). As a result, the specific activity of the PEGylated SOD was not associated with the molecular weight of PEG and was only determined by the modification rate of the lysine residues. On the other hand, bioactive proteins such as TNF-alpha, IL-6 and LIF require binding to macromolecular receptors for the exertion of activity. In such bioactive proteins, in addition to a decrease in the specific activity due to modification of the lysine residues, consideration should be given to the inhibition of activity caused by the steric hindrance of PEG. In most cases, the PEGylation of proteins is non-specific and targets all lysine residues in the protein, some of which may be in or near the active site. To overcome this drawback, Pastan *et al.* attempted to carry out site-specific PEGylation of mutant PE molecules that were engineered to contain one or two cysteine residues on their surface [54,55]. PEG was attached to these residues by free thiol chemistry. However, this approach proved to be unsuccessful due to a low yield of PEGylated immunotoxin and a significant loss in activity. Recently, different approaches to site-specific PEGylation have been chosen to overcome these problems of PEGylation using anti-Tac(Fv)-PE38 as a model recombinant immunotoxin. To keep the antigen-binding, translocation and ADP-ribosylation activities intact, a mutant anti-Tac(Fv)-PE38, containing a single cysteine in the peptide connector that attaches the Fv moiety to the toxin, was prepared. Subsequently, site-specific modification of the mutant LMB-2 with PEG-maleimide, via the formation of a thioether bond, was carried out.

In anti-Tac(Fv)-PE38, the Fv portion of the anti-Tac antibody is linked to PE38 by a peptide connector (ASGGPE). To prevent loss of the antigen-binding, translocation and ADP-ribosylation functions of anti-Tac(Fv)-PE38 that are necessary for its specific cytotoxic activity against CD25+ tumor cells, we prepared a mutant form of anti-Tac(Fv)-PE38 with one cysteine in the peptide (ASGCPE) that connects the Fv to PE38. Site-specific PEGylation was carried out at this cysteine using 20-kDa and 5-kDa of PEG. Both types of PEGylated anti-Tac(Fv)-PE38 had similar cytotoxic activities compared to the unmodified anti-Tac(Fv)-PE38. The anti-tumor activities of both types of PEGylated mLMB-2s *in vivo* were markedly higher than the native anti-Tac(Fv)-PE38. Additionally, the plasma half-life of the unmodified anti-Tac(Fv)-PE38 was about 13 min. In contrast, the serum concentration profiles of both PEGylated anti-Tac(Fv)-PE38 showed monoexponential elimination curves. The plasma half-life of PEG5K-anti-Tac(Fv)-PE38 and PEG20K-anti-Tac(Fv)-PE38 increased by about 5-fold and 8-fold, respectively. Native anti-Tac(Fv)-PE38 markedly induced anti-(anti-Tac(Fv)-PE38) IgG antibodies in mice. In contrast, the immunogenicity of both PEGylated LMB-2s was found to be much lower. Therefore, it has been shown that site- specific PEGylation of recombinant immunotoxin increases its stability, blood-residency time and anti-tumor activity, while decreasing its non-specific toxicity and immunogenicity. The overall therapeutic window increased over 20-fold. These results have important clinical implications for the use of immunotoxins in patients. The approach used for improving the action of anti-Tac(Fv)-PE38 should be applicable to other recombinant immunotoxins as well and may increase their activity.

6. STRATEGY OF TUMOR VASCULAR TARGETING

Targeting therapy using immunoconjugates and immunotoxins composed of a monoclonal antibody to tumor cells and an anti-tumor antibiotics/toxin is attractive. However, the use of a monoclonal antibody against tumor cells themselves has often been ineffective due to their poor

penetration into tumor tissue [6]. As mentioned above, recombinant immunotoxins, have shown great anti-tumor effects to hematopoietic malignancies in Phase I trials, but there has been no significant therapeutic response to solid tumors. This can be attributed to their short plasma half-lives and insufficient tumor accumulation whereas, by using whole monoclonal antibodies, it is known that the distribution of recombinant immunotoxins in tumors is much higher than that of immunoconjugates and immunotoxins [8,56]. Furthermore, targeting tumor cells requires monoclonal antibodies against different types of tumor cells, because the antigens of tumor cells are heterogeneous. Tumor growth is dependent on new blood vessel formation to supply nutrients and oxygen [57,58]. The vasculature that is created by angiogenesis, as the tumor develops, is reported to be similar among various tumor types. Additionally, tumor vasculature has properties that differ from those of normal vasculature, such as enhanced permeability, suppressed leukocyte adhesion and high sensitivity to TNF-alpha [59-61]. These anatomical, morphological and behavioral differences between blood vessels in tumor and in normal tissues suggest that antigenic differences would be induced on endothelial cells by the tumor microenvironment. Recent reports indicate a higher expression of some molecules on tumor vascular endothelium than on normal endothelium [62-64]. These molecules include endoglin [62], endosialin and aminopeptidase N [63,64] and are considered suitable candidates for tumor antibody therapy, since the antibody can freely access the target regardless of vascular permeability. Furthermore, destruction of the tumor vascular endothelium can cause irreversible clotting, resulting in the formation of an occlusive thrombus that would halt blood flow and cause effective tumor regression (Fig. 3). However, a specific monoclonal antibody against TEC without some monoclonal antibodies to overexpressed antigens, such as VEGF-receptor, had not been previously prepared. Furthermore, neither isolation nor culture of TEC, which may be an immune source, has been performed. In the next section, in order to develop the tumor targeting therapy, we initially described the method of isolation of vascular endothelial cells from tumor tissues, and then indicate its usefulness as tumor therapy.

7. ISOLATION AND PROPERTIES OF TUMOR-DERIVED ENDOTHELIAL CELLS

It is believed that tumor and stroma cells, directly or indirectly, affect the properties of endothelial cells in tumor tissues. It is now well-established that great heterogeneity exists between not only endothelial cells in macro and microvessels but also among microvessels and endothelial cells from different tissues [65-73]. Brain microvessel endothelial cells form a blood-brain barrier through which macromolecules cannot pass. Liver and spleen microvessels, known as discontinuous capillaries, possess open cellular junctions that allow the passage of macromolecules. Blood vessels in tumors also show significant differences from those of normal tissues, in terms of structure and function. Many investigators have reported that tumor vessels are more permeable than normal tissue vessels. The various tissue-derived endothelial cells that have been cultured and examined to date include those from the human umbilical vein, human adrenal capillary, rat cerebral microvessels and bovine retinal microvessels. However, a method to isolate and culture TEC had not yet been developed.

We isolated TEC from KMT-17 fibrosarcoma-bearing rats by Percoll's density gradient centrifugation method. We examined whether the isolated TEC maintained the *in vivo* properties of tumor-derived vasculature. Tumor vessels are more permeable than normal tissue vessels *in vivo*. TEC monolayers showed greater permeability than the aortic, venous and epididymal fat-derived endothelial monolayers by FITC-dextran (molecular weight 70,000) diffusion (Table 1). Leukocyte adhesion to tumor vessel endothelium is known to be often lower than that to normal tissue vessel endothelium (Table 2). We examined this feature in the isolated TEC, and found that leukocyte adhesion to TEC was reduced compared to that of rat epididymal-fat pad derived capillary endothelial cells (FCEC).

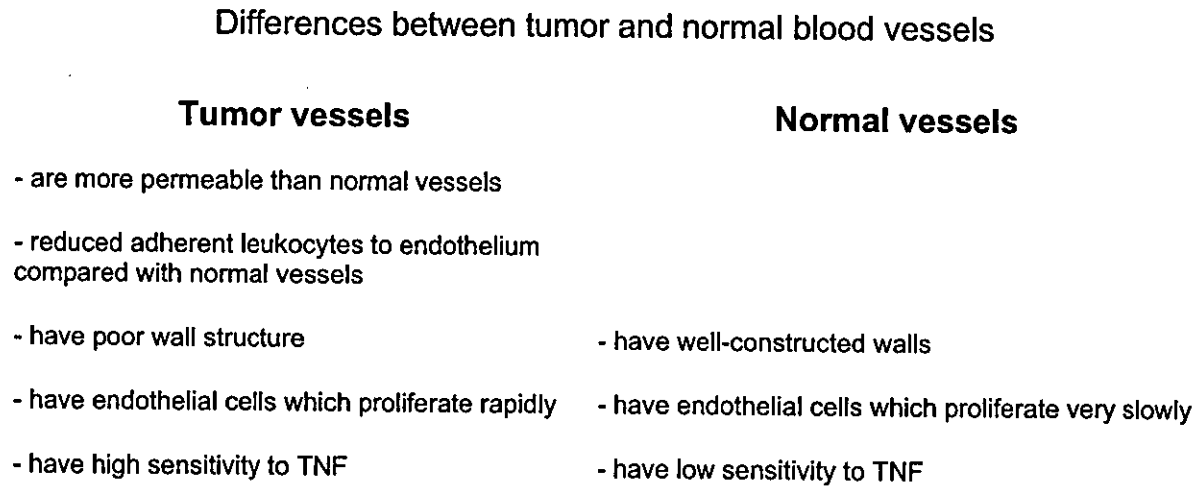

Fig. (3). Targeting therapy of cancer with monoclonal antibody against tumor derived endothelial cells.

Table. 1. Permeability of Rat Endothelial Monolayers to FITC-dextran. Rat Aortic, Vena Cava, Epididymal Fat Capillary and Tumor-derived Endothelial Cells were Cultured on the Permeation Chamber. When the Cells were Confluent, the Permeability of FITC-dextran (Molecular Weight 70,000) was Determined. Mean±SD. N=4

Source	Permeability coefficient ($\times 10^{-3}$cm/h)
Aorta	17.23 ± 2.52
Vena cava	8.43 ± 1.96
Fat capillary	19.49 ± 3.57
Tumor capillary	41.10 ± 3.99

Table. 2. Number of Leukocytes Adhering to Endothelial Cells. Rat Epididymal Fat-derived Endothelial Cells (FCEC) and Tumor-derived Endothelial Cells (TEC) were Cultured on a 96-well Tissue Culture Plate. When the Cells were Confluent, Rat Leukocyte Adhesion to Endothelial Cells was Assayed. a) P<0.001 Compared from FCEC by Students' t-Test. Mean±SD. N=4

	$\times 10^5$cells/cm^2	X10^5cells/10^5cells
FCEC	1.58 ± 0.12	2.83 ± 0.22
TEC	0.86 ± 0.14[a)]	1.75 ± 0.28[a)]

Various tissue-derived endothelial cells have been cultured, and it is very important to determine whether the isolated cells retain the properties of the tissues from which they were derived. Endothelial cells present not only tissue characteristics but also properties specific to the type of vessel from which they are derived i.e., aorta, vena cava or capillaries. *In vivo*, histamine induces plasma protein leakage in the venous and capillary vessels; it also increases venous and capillary-derived endothelial permeability in culture systems. In contrast, histamine has been shown to decrease bovine aortic endothelial cell permeability. These findings suggest that it is necessary to study endothelial cells derived from the specific tissue of interest to obtain valid results. Primary cultures of TEC showed hyperpermeability to macromolecule diffusion and low leukocyte adhesion, properties similar to tumor vascular endothelium *in vivo*.

It is known that the *in vivo* features of cells obtained by primary culture gradually disappear after repeated cell passages. Therefore, we examined the number of cell passages during which the TEC maintained the features of tumor vessels, using permeability and leukocyte adhesion as indices. As a result, TEC maintained the features of tumor vasculature until the first passage, but the features disappeared after the fourth passage. This may be due to the discontinuation of various factors obtained from tumor cells. Therefore, we used cells obtained by primary culture or the first cell passage for all our experiments. Thus, it is very likely that the TEC isolated in this study can be utilized as an immune source for preparing a monoclonal antibody against tumor-tissue endothelium.

8. PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO TUMOR-DERIVED ENDOTHELIAL CELLS

To develop antibody-based tumor vascular targeting therapy for solid tumors, mice were immunized with TEC by passive and active immunization methods using TEC in which the *in vivo* features of tumor vasculature was maintained [74-76]. The mice were initially immunized with membrane protein components extracted from normal FCEC. After the antiserum collected from these mice was administered to other mice, these mice were immunized with vesicles from TEC to prepare hybridomas that produce a specific antibody against TEC. The specificity of the antibody to TEC was evaluated by ELISA using TEC as a solid antigen and immunostaining using frozen tissue sections of the parent rat tumor (KMT-17) tissue. As a result, five kinds of tumor tissue blood vessel-specific monoclonal antibodies were obtained. Of these, we will describe a monoclonal antibody that was named TES-23.

First, we examined tissue distribution, 1 h after intravenous administration of TES-23, using KMT-17 tumor-bearing rats. A negative antibody, MOPC, did not accumulate in tumor tissues. However, TES-23 markedly accumulated in tumor tissues within a short period; only 1 h (Fig. 4). Generally, when an antibody against tumor cells

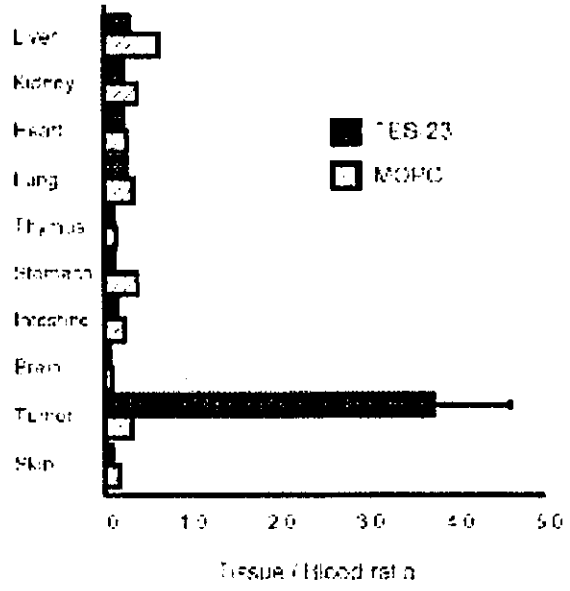

Fig. (4). Tissue distribution of ^{125}I-Labelled TES-23 in QKAH rats bearing KMT-17 fibrosarcoma.
100ng of radiolabelled TES-23 of MOPC were injected intravenously into tumor-bearing rats. One hour later the rats were anaesthetized and exsanguinated via the abdominal aorta. Each organ was removed and its radioactivity was counted by an auto gamma counter. Each value shown is the Mean ± SE for four animals.

themselves is used, antibodies do not accumulate in tumor tissues within 1 h of administration, and only a 2 or 3-fold amount of antibodies transfer to tumor tissues compared to that of negative antibodies 24 h after administration. Considering this finding, our results strongly suggest the efficacy of missile therapy targeted against tumor vasculature. Additionally, we evaluated, in detail, the immunostaining of KMT-17 tumor tissue sections with TES-23. It was suggested that TES-23 might recognize endothelial cells of nourishing blood vessels and neogenetic blood vessels in tumor tissues. Therefore, the specificity of TES-23 to TEC was suggested.

Subsequently, we examined the *in vivo* antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) -mediated anti-tumor effects of administrating TES-23 antibodies alone. KMT-17 fibrosarcoma was intracutaneously inoculated in rats. One mg/rat (about 5 mg/kg) of TES-23 antibodies alone were intravenously administered for 5 days starting from 4 days after tumor inoculation. Cisplatin, which was used as a positive control, markedly inhibited tumor proliferation. Marked anti-tumor effects were observed in the TES-23-treated group compared to those in the control group. Furthermore, in the group treated with MOPC, no anti-tumor effects were observed. In contrast, marked weight loss was observed in the group treated with 1.2 mg of cisplatin. However, no weight loss was observed in the TES-23-treated group. These findings suggest that TES-23 alone inhibits tumor proliferation without serious side effects.

Hematoxylin and eosin staining of tumor tissue sections, 24 h after intravenous injection of 1 mg/rat of TES-23, was performed to clarify the usefulness of TES-23 as a missile molecule. In rats treated with the control antibody (MOPC), spontaneous necrosis was observed only at the center of the tumor focus. However, in the TES-23-treated group, extensive tissue necrosis was observed. In addition, degeneration, necrosis and exfoliation of TEC was observed in the TES-23-treated group. Furthermore, degeneration of tumor cells was observed in the TES-23-treated group. From the result of Elastica Van Gieson's staining, swelling of TEC was observed whereas no changes were observed in the control antibody-treated group. Additionally, fibrin thrombus formation was observed in the TES-23-treated group by phosphotungstic acid hematoxylin staining. No marked changes were observed in normal tissues after TES-23 administration. These results suggest that the anti-tumor effects of TES-23 administration involved the following mechanism: CDC or ADCC was induced by specific binding of TES-23 to the TEC, causing TEC injury and promoting thrombus formation and thus cutting off the lifeline of tumor cells. Therefore, TES-23 is expected to be a useful missile molecule.

9. PREPARATION AND CHARACTERIZATION OF THE IMMUNOCONJUGATE CONSISTING OF TES-23 AND NEOCARZINOSTATIN

We prepared an immunoconjugate (TES-23-NCS) consisting of TES-23 and a protein anti-tumor drug, neocarzinostatin (NCS), and applied TES-23-NCS to tumor vascular targeting therapy [77-79]. The TES-23-NCS conjugate was prepared by cross-linking the Fc site of TES-23 with the apoprotein site that was not associated with the site of NCS activity. Therefore, the immunoconjugate (TES-23-NCS) synthesized by this method completely sustained both the antibody titer and NCS activity. As described above, TES-23 was obtained using rat KMT-17-derived TEC as an immune source. Therefore, we initially examined the effects of the TES-23-NCS conjugate on KMT-17 solid tumors for primary screening. TES-23-NCS conjugate was administered through the tail vein. In the group treated with the TES-23-NCS conjugate, marked anti-tumor effects on rat KMT-17 solid tumor were observed. A conjugate consisting of MOPC and NCS did not show any efficacy. Administration of the TES-23-NCS conjugate resulted in tumor hemorrhagic necrosis, suggesting that the anti-tumor effects of this conjugate were associated not only with improved blood retention but also with tumor regression related to the decay of blood vessels. Subsequently, the *in vivo* distribution was examined to clarify the mechanism involved in the anti-tumor effects. One hour after intravenous administration, TES-23 and the TES-23-NCS conjugate markedly accumulated in rat KMT-17 tumor tissues. .

TES-23 will be a highly favorable missile molecule if specific molecules that are common among animal species or tumor types appear on TEC and can be recognized by TES-23. To evaluate the efficacy of TES-23 on various tumors derived from several animal species, we initially examined the anti-tumor effects of the TES-23-NCS conjugate on murine Meth-A fibrosarcoma (Fig. **5** and Table **3**). The administration of a 10-fold dose (500 μg/kg) of NCS alone did not achieve tumor retraction, although tumor proliferation was slightly inhibited. Furthermore, efficacy was not obtained in groups treated with TES-23 alone (320 μg/kg) or a simple mixture of TES-23 (320 μg/kg) and NCS (50 μg/kg), whereas 5 mg/kg of TES-23 alone showed anti-tumor activity to rat solid tumor, as mentioned above. However, in the group treated with the TES-23-NCS conjugate (TES-23: 320 μg/kg, NCS: 50 μg/kg), marked anti-tumor effects with tumor hemorrhagic necrosis were obtained without any serious side effects. However, the efficacy disappeared when free TES-23 was simultaneously administered. A conjugate consisting of MOPC and NCS did not show any efficacy. Therefore, we found that the TES-23-NCS conjugate showed potent anti-tumor effects not only on rat KMT-17 but also on murine Meth-A without causing serious side effects. Next, we examined the pharmacokinetics of TES-23 in Meth-A tumor-bearing mice after intravenous administration (Fig. **6**). Within a short period of 1 h, TES-23 showed tumor tissue accumulation that was 50-fold that of MOPC. TES-23 did not accumulate in normal tissues. Additionally, tumor vasculature accumulation of TES-23 was completely inhibited by pre-treatment with unconjugated TES-23. Therefore, the anti-tumor effects of the TES-23-NCS conjugate on murine Meth-A may have been obtained because the TES-23-NCS conjugate specifically accumulated in tumor vasculature, as demonstrated in the case of rat KMT-17. These findings suggest that TES-23 recognizes not only rat tumor vasculature but also murine Meth-A tumor vasculature. In addition, murine tumors other than Meth-A were similarly

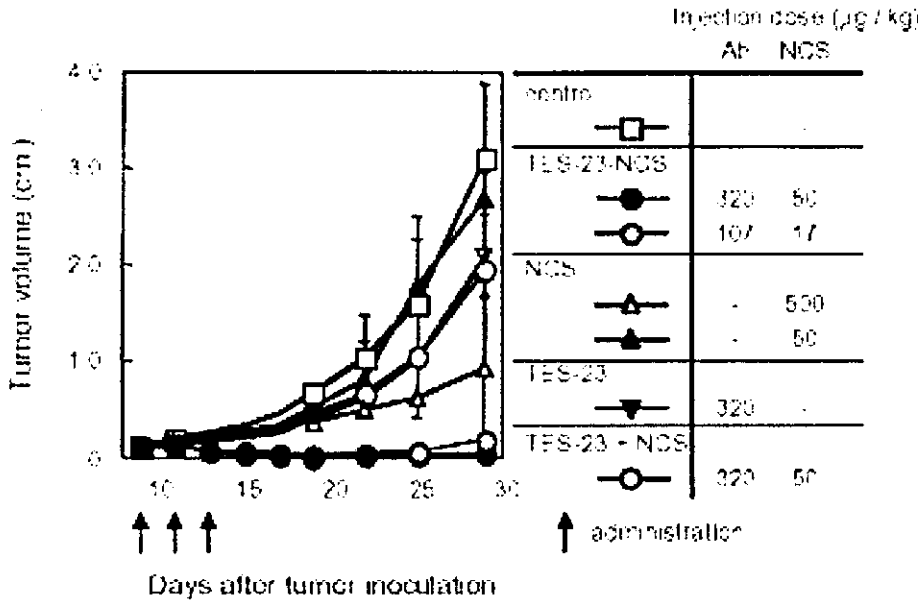

Fig. (5). Antitumor effects of TES-23-NCS on Meth-A Solid Tumor in BALB/c Mice.
Meth-A cells were implanted intradermally in the abdomen of BALB/c mice. TES-23-NCS conjugates or control was given intravenously on day9, 11, and 13 after tumor inoculation. Treatments are indicated by the arrows. Each value shown is the Mean ± SE for four animals.

Table. 3. Antitumor Effects of TES-23-NCS in Terms of Survival Days after Meth-A Tumor Inoculation

a) Groups of Four Mice Received Each Treatment Intravenously at day 9, 11, and 13

b) Days After Tumor Inoculation (Mean±SE).

c) Complete Regression was Defined as No Tumor Regrowth for More than 120 Days

d) Significant Difference from Control Group ($p<0.05$)

	Injection dose (μg/kg)[a]		Survival time (days)[b]		Complete regression[c]
	Ab	NCS			
control	-	-	53± 6	(39, 48, 59, 65)	0/4
TES-23-NCS	320	50	103±19[d]	(53, 120<, 120<, 120<)	3/4
	107	17	95±17[d]	(67, 72, 120<, 120<)	2/4
TES-23-NCS	107	17	46± 5	(34, 46, 50, 55)	0/4
+TES-23 (1070 μg/kg)	320	50	50± 3	(45, 49, 50, 57)	0/4
MOPC-NCS	107	17	45± 3	(38, 46, 47, 48)	0/4
NCS	-	500	63±25	(15, 48, 68, 120<)	1/4
	-	50	49± 6	(31, 45, 51, 52)	0/4
TES-23	320	-	45± 4	(39, 39, 46, 53)	0/4
TES-23+NCS	320	50	51± 3	(46, 49, 50, 57)	0/4

examined. We examined tissue accumulation of TES-23 in Colon 26 adenocarcinoma or S-180 sarcoma-bearing mice. As observed in Meth-A tumor-bearing mice, TES-23 specifically accumulated in these murine tumors. In a human tumor-implanted model, TES-23 also specifically accumulated in tumor tissues at a level 30-fold that of MOPC. In this experimental system, tumor tissue blood vessels were derived from mice. However, this result suggests that common specific molecules recognized by TES-23 may also be induced in TEC in humans. Therefore, it is suggested that there may be specific molecules that are common among various animal species or tumor types on